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Uncovering behavioural diversity amongst high-strength *Pseudomonas* spp. surfactants at the limit of liquid surface tension reduction

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Abstract (197/200 words)

Bacterial bio-surfactants have a wide range of biological functions and biotechnological applications. Previous analyses had suggested a limit to their reduction of aqueous liquid surface tensions (γ_{Min}), and here we confirm this in an analysis of 25 *Pseudomonas* spp. strains isolated from soil which produce high-strength surfactants that reduce surface tensions to 25.2 ± 0.1 – 26.5 ± 0.2 mN.m^{-1} (the surface tension of sterile growth medium and pure water was 52.9 ± 0.4 mN.m^{-1} and 72.1 ± 1.2 mN.m^{-1}, respectively). Comparisons of culture supernatants produced using different growth media and semi-purified samples indicate that the limit of 24.2 – 24.7 mN.m^{-1} is not greatly influenced by culture conditions, pH or NaCl concentrations. We have used foam, emulsion and oil-displacement behavioural assays as a simple and cost-effective proxy for in-depth biochemical characterisation, and these suggest there is significant structural diversity amongst these surfactants which may reflect different biological functions and offer new biotechnological opportunities. Finally, we obtained a draft genome for the strain producing the highest-strength surfactant, and identified a cluster of non-ribosomal protein synthase genes which may produce a cyclic-lipopeptide (CLP)–like surfactant. Further investigation of this group of related bacteria recovered from the same site will allow a better understanding of the significance of the great variety of surfactants produced by bacterial communities found in soil and elsewhere.

Keywords : *Pseudomonas*, surfactant, limit to liquid surface activity, cyclic lipopeptide, non-ribosomal protein synthase.

One sentence summary : (29 / 30 words) Bacterial bio-surfactants appear to have a limit to aqueous liquid surface tension activity of ~24 mN.m^{-1} which is approached by a number of pseudomonad surfactants showing significant behavioural diversity.

1. Introduction

Bio-surfactants produced by bacteria are surface-active agents having a wide range of biological activities including involvement in the solubilisation of hydrophobic substrates, co-ordinated growth and differentiation, cell motility, surface attachment and biofilm-development, suppression of competitors and protection from predators, immune modulation and virulence, rotting of plant tissues, causing fungal hyphae swelling and the lysis of oomycete zoospores (Ron & Rosenberg, 2001; Abdel-Mawgoud et al. 2010; Raaijmakers *et al.* 2010). These compounds also have many applications in cosmetic, food, medical, pharmaceutical, oil and bioremediation technology where new high-strength surfactants are constantly in demand as detergents, wetting and foaming agents, emulsifiers and dispersants (Franzetti *et al.* 2010; Marchant & Banat, 2012; Gudiña *et al.* 2013; Souza *et al.* 2014;

Inès & Dhouha 2015). The activity of surfactants depends on their amphiphilic nature, and a number of different structural classes of surfactants are produced by bacteria (Desai & Banat, 1997), including cyclic-lipopeptides and rhamnolipids (Abdel-Mawgoud *et al.* 2010; Raaijmakers *et al*. 2010).

However, the relationship between surfactant activity, biological function (or role) and structural diversity remains poorly understood; we need to separate activity resulting from the fundamental biophysical properties of surfactants from those biological activities that provide the surfactant–producer with a selective advantage. For example, very few bacteria would have a selective advantage in lysing erythrocytes, yet this is a common assay for surfactant production (e.g. Youssef *et al.* 2004; Afshar *et al*. 2008). Similarly, it is not clear how much of the observed structural diversity amongst surfactants is relevant or redundant. Furthermore, in complex soil or plant-associated communities where different bacteria are capable of producing a range of surfactants, are these treated as public goods benefiting the whole community or does this represent intra-community conflict and competition?

Our research has focussed on assessing bacterial surfactant strengths and behavioural diversity within the *Pseudomonas* genus using behavioural assays as a simple and cost-effective proxy for the in-depth biochemical characterisation required to determine structural diversity (Fechtner *et al*. 2017). This genus includes plant and mushroom pathogens that use surfactants to rot tissues as well as many surfactant-producing soil and plant-associated strains found in complex communities where the suppression of the growth of competitors and protection from predators may be particularly important; in addition, surfactants are also required for swarming motility and biofilm maturation (Raaijmakers *et al.* 2010). Recent investigations of high-strength surfactants produced by pseudomonads and other bacteria have suggested that there is a limit (γ_{Min}) to the extent surfactants can reduce aqueous liquid surface tension of 24.16 – 24.24 $mN.m^{-1}$ (Fechtner *et al.* 2011; Mohammed *et al.* 2015) and the biological basis for this is probably the need to minimise self–damage to the producing cells (Fechtner *et al.* 2017). To put this into context, the surface tension of water at 20°C is 72.8 $mN.m^{-1}$ (Vargaftik *et al*., 1983) while the sterile media used in these predictions have surface tensions of between 47.0 – 59.6 $mN.m^{-1}$ (Fechtner *et al.* 2011; Mohammed *et al.* 2015).

In this work we want to test the robustness of the prediction by investigating surfactant production amongst a collection of pseudomonads isolated from the same soil community, to determine whether culture and buffer conditions significantly alter liquid surface tension measurements and γ_{Min}, and to assess the structural diversity amongst the high-strength surfactants produced by these strains which may represent a valuable resource for future biotechnological exploitation.

2. Materials and Methods

2.1. Bacterial isolation and cultivation

Pseudomonas spp. or *Pseudomonas*–like strains were isolated from samples taken from bulk soil underlying a section of managed grass lawn at the Dundee Botanic gardens (Dundee, UK) in February and April, 2015. Bacteria were isolated using selective agar (PSA-CFC; Oxoid, UK) spread with soil suspension dilutions and incubated under aerobic conditions for 2 – 3 days at 20 – 22 °C. Colony material re-suspended in deionised water (DI) was used to test for surfactant production using the drop collapse assay on petri dish lids after Persson & Molin (1987) and then confirmed by quantitative tensiometry of modified King's B (KB*; Kuśmierska & Spiers, 2017) culture supernatants (see below). Twenty-five surfactant-producing strains (Dundee Botanic Garden (DBG) strains 1 – 25) plus 5 randomly chosen drop collapse-negative strains (DBG strains c1 – c5) were retained for further investigation and stored at -80 °C in 15% (v/v) glycerol. Over-night KB* or minimal medium containing 20 mM glucose (M9-Glu; Fechtner *et al.* 2011) cultures incubated with shaking at 28 °C were used to prepare samples for testing as required.

2.2. Strain characterisation and identification

Phenotypes were determined using biochemical, growth and behaviour–based assays at 20 – 22 °C after Robertson *et al.* (2013) (see Supplementary Information for further details) and Hierarchical cluster analysis (HCA) used to group strains on the basis of similarity after Robertson *et al.* (2013) and Mohammed *et al.* (2015). Key strains were further analysed by metabolic profiling using API 20e cards (BioMérieux, UK) and partial 16S rDNA sequencing to determine genus-level identification (see Supplementary Information for further details).

2.3. Surfactant behaviour and surface tension measurements

24 h KB* cultures were used to investigate surfactant behaviours using emulsion, foam stability and oil displacement assays at 20 – 22 °C after Coffmann & Garcia (1977), Cooper & Goldenberg, (1987) and Morikawa *et al*. (1993) (see Supplementary Information for further details) and Hierarchical cluster analysis (HCA) used to cluster surfactant behaviours on the basis of similarity. For the oil displacement assays (also known as oil spreading assays), Mineral oil, Vegetable oil, Used lubricating oil (ULO) and Diesel were over-laid onto DI water (pH 6), 200 mM NaCl (pH 6) and 50 mM Tris (pH 8) solutions. Surfactants were semi-purified from 24 h KB* cultures by an acid precipitation method adapted from De Souza *et al.* (2003) and re-suspended in DI water to test critical micelle concentrations, pH and NaCl surface tension profiles (see Supplementary Information for further details). Quantitative tensiometry of semi-purified surfactant solutions and cell-free 24 h KB* or M9-Glu culture supernatants were performed using a Krüss K100 Mk2 Tensiometer at 20°C after Koza *et*

al. (2009) and mean surface tension measurements are rounded up to one decimal place. In these assays, the surface tension of pure water was 72.1 ± 1.2 $mN.m^{-1}$, and the surface tension of sterile KB* and M9Glu culture media was 52.9 ± 0.4 and 70.7 ± 0.7 $mN.m^{-1}$, respectively.

2.4. Statistical analyses

Experiments were performed with replicates and means with standard errors (SE) are shown where appropriate. Data were assumed to be Normally distributed and were examined using JMP v12 statistical software (SAS Institute Inc.) with comparisons of means performed using Student's and matched pairs *t*-tests (*t*), one-way ANOVA (*F*) models with Tukey-Kramer HSD (*q**) *post hoc* tests and correlations (*r*) examined by multivariate analysis. Hierarchical cluster analysis (HCA) using the Ward Method with equal weightings was used to investigate similarities between strain phenotypes and surfactant behaviours after Robertson *et al.* (2013) and Mohammed *et al.* (2015). Analyses based on general linear models (GLMs) were used to investigate surface tension and oil displacement data with effects further examined using LSMeans Differences Tukey HSD (*Q*) tests (see Suppl. Table S1 for model details, co-variates and effects tests). The minimum liquid surface tension (γ_{Min}) was determined by Individual distribution identification (IDI) after Fechtner *et al.* (2011) using mean surface tension data and based on the lowest Anderson-Darling (*AD*) goodness of fit test value using MINITAB v1.5 statistical software (Minitab Ltd, UK).

2.5. DBG-1 draft genome and identification of possible surfactant synthesis genes

The DBG-1 draft genome was determined using the microbial sequencing and strain repository service MicrobesNG (microbesng.uk; Birmingham, UK) and trimmed reads and fasta files are available on request. Sequencing was performed on Illumina MiSeq and HiSeq 2500 platforms using 2x 250 bp pair-end reads, and data put through a standard analysis pipeline for assembly and quality analysis (see microbesng.uk for further details). A mean coverage of 42.6x was achieved with 656,944 reads, producing a draft genome of 6,860,106 bp comprised of 122 contigs of which the largest was 657,704 bp and a GC ratio of 58.9%. A total of 6,082 coding sequences (CDS) were predicted within contigs, with an average length of 976 bp and density of 0.89 per kb, and annotations provided where possible by automated BLAST analyses. A total of 69 tRNA genes identified, though no rRNA genes were found including the 16S rDNA gene required for species–level identification. Read mapping suggests that this genome is most closely related to the *P. fluorescens* species which is consistent with our isolation and selection of the strain as a fluorescent pseudomonad.

CDS annotations associated with non-ribosome protein synthases (NRPS) were inspected manually and confirmed by NCBI/NLM BLASTP against non-redundant GenBank CDS translations, PDB, SwissProt, PIR and PRF databases (blast.ncbi.nlm.nih.gov/Blast.cgi). The draft DBG-1 genome was

also submitted to antiSMASH (antismash.secondarymetabolites.org; bacterial version; Weber *et al.* 2015) to further characterise NRPS homologues and predict possible products.

3. Results and Discussion

3.1. Isolation of Pseudomonas spp. expressing strong surfactants which significantly lower liquid surface tension

We isolated a collection of *Pseudomonas* spp. or *Pseudomonas*–like bacteria from soil that produced high–strength surface active agents or surfactants when incubated in KB* cultures for 24 h, and from a statistically-homogeneous Tukey-Kramer HSD group ($q^* = 3.970$; $\alpha = 0.05$) chose the 25 strains producing the lowest surface tensions for further analysis (DBG-1 – 25; Figure 1). Although these showed similar surface tension reducing activity ranging between 25.2 ± 0.1 – 26.5 ± 0.2 $mN.m^{-1}$, a comparison of strain phenotypes by Hierarchical cluster analysis (HCA), which also included control strains not producing surfactants under the conditions used here (DBG-c1 – 5), indicated that most could be differentiated by one or more colony morphology, enzyme and siderophore expression, antibiotics and mercury sensitivity, salt and high temperature tolerance, and motility assays (Figure 2; see Suppl. Table S2 for the ordinal data-set), with little evidence of biological replication (i.e. the isolation of the same strain more than once). Further testing of key strains using metabolic profiling and partial 16S rDNA sequencing suggest that most are probably *Pseudomonas* spp. (see Suppl. Table S3 for putative identifications). This collection of phenotypically diverse pseudomonads producing high-strength surfactants provided us with an opportunity to test the robustness of earlier predictions of the minimum limit (γ_{Min}) to liquid surface tension reduction achieved by bacterial surfactants, and then to examine the degree of structural diversity within a group of high-strength surfactants.

3.2. Testing the predicted limit to liquid surface tension reduction

We used Individual distribution identification (IDI) to predict γ_{Min} following the method established by Fechtner *et al.* (2011). The best fit for the mean surface tension data was found using the 3-parameter Log-logistic distribution ($AD = 0.721$) and produced a value of 24.7 $mN.m^{-1}$ for γ_{Min} similar to the earlier predictions of around 24.2 $mN.m^{-1}$ determined for a number of different groups of bacteria (Fechtner *et al.* 2011; Mohammed *et al.* 2015). To put this range of predictions into context, it is lower than the standard errors in our measurement of the surface tension of DI water at 20°C which we undertake as an internal control (72.1 ± 1.2 $mN.m^{-1}$) and less than the change in surface tension of water between 20 °C and 25 °C (72.8 and 72.0 $mN.m^{-1}$, respectively; Vargaftik *et al*., 1983) (in comparison, the surface tension of an 80% (w/w) solution of ethanol at 20°C is 24.3 $mN.m^{-1}$;

Vázquez *et al*., 1995). This suggests that these predictions are centring on a common value for γ_{Min}; however, differences in pH and solute concentrations in culture media and resuspension buffers might be expected to alter surface tension measurements and γ_{Min} in an assay-dependent manner. We decided to explore this further by comparing KB* culture supernatant surface tensions with measurements taken from M9-Glu minimal medium and semi-purified surfactants re-suspended in DI water.

We obtained a slightly higher γ_{Min} prediction from the M9-Glu cell-free culture supernatants of 25.0 $mN.m^{-1}$ using a 3-parameter Gamma distribution ($AD = 0.917$). However, although there was no significant correlation between KB* and M9-Glu strain means ($p = 0.27$), there were significant differences between pairs ($t = -3.7527$, $p = 0.0003$) with 7 strains showing more activity and 6 strains less activity in M9-Glu than might have been expected when compared to KB* (see Figure S1 for a comparison of KB* and M9-Glu ST means). This suggests that the surfactants produced by some strains were differentially sensitive to the media or final culture supernatant used to determine surface tension measurements.

In order to investigate this further, we semi-purified surfactants produced by 14 strains representing the range of surfactant strengths produced by this collection (DBG-1 – 5, 7, 10, 14 – 16, 20, 21, and 25). We progressively diluted these samples with DI water to demonstrate that in each case surfactants were produced in KB* cultures above the critical micelle concentration (CMC), and that minor differences in concentration could not explain the differences in surface tension seen between strains or between KB*, M9-Glu and re-suspended surfactant surface tension measurements. We then modelled surface tension measurements using a general linear model (GLM), and found that strain and assay environment were significant effects (GLM model I, $p < 0.0001$; see Suppl. Table S1 for further details), with the re-suspended surfactant surface tensions significantly higher than both culture supernatant measurements (by approx. 2.5 $mN.m^{-1}$; $q^* = 2.4973$, $\alpha = 0.05$). Collectively these findings suggest that the liquid surface tension produced by these surfactants are effected by the environment in which they are measured (e.g. by differences in pH, solute concentrations and the presence of other compounds which may differentially interact with each surfactant), but despite this, variations in surfactant concentrations and environment effects do not explain the γ_{Min} limit of 24 – 25 mN^{-1} to surface tension reducing activity.

We have proposed that the γ_{Min} limit is likely to reflect the extent of self-damage surfactant-producing cells can tolerate (Fechtner *et al*. 2017), as surfactants are known to have a toxic effect and cause the loss of lipopolysaccharide (LPS) in some bacteria, and more generally damage cell membranes and cause cell lysis in a range of prokaryote and eukaryote cells (Raaijmakers *et al*. 2006 & 2010; Franzetti *et al*. 2010; Inès & Dhouha 2015). Such self-damage is similar in nature to the effect of

antibiotics, but whereas protective features such as altered targets and efflux pumps have been identified in many antibiotic-producing or resistant organisms (Cundliffe 1989), nothing is known about how bacterial cells might protect themselves from damage caused by the surfactants they produce.

In contrast to bacterial surfactants, synthetic hydrocarbon surfactants can reduce liquid surface tensions to approx. 24 $mN.m^{-1}$, while fluorocarbon and silicon surfactants can reduce surface tensions to as low as 13.7 $mN.m^{-1}$ (Czajka *et al.* 2015). This may represent the physical-chemical limit of liquid surface tension reduction by surfactants which is a direct consequence of the hydrophobic tail CH_3/CH_2 ratio per hydrophilic head-group of these amphiphilic compounds, with synthetic 'hedgehog' surfactants more densely packed with CH_3/CH_2 groups than linear-chain surfactants (Czajka *et al*. 2015) such as the bacterial CLPs and rhamnolipids with 1 – 2 tail chains per head group (Abdel-Mawgoud *et al.* 2010; Raaijmakers *et al*. 2010).

3.3. Significant behavioural variation exists within these high-strength surfactants

We have also looked at surfactant behaviours as a proxy for the structural differences found between these compounds, as this is of general interest in determining the diversity of surfactant production and ecological roles these compounds may play within soil communities, as well as potential applications in biotechnology. We used quantitative emulsion, foam stability and oil displacement assays to generate a behavioural data set that we then investigated by HCA to visualise similarities in surfactant behaviours (these assays can all be modified by changing pH, NaCl concentration, temperature, etc. to reveal further behavioural differences; e.g. Zhang & Miller 1992; Morikawa *et al*. 2000; Prieto *et al.* 2008; Rocha e Silva *et al*. 2014; Balan *et al*. 2016; Liu et *al.* 2016).

Preliminary testing of individual assays by one-way ANOVA found significant differences between surfactant–producing strains as well as between these and the control strains (data not shown). Further HCA of various combinations of assays showed that assay type, oil and aqueous layer conditions resulted in subtly different clustering of surfactants, and in modelling the oil displacement data alone, strain, oil-type, and aqueous layer conditions were all found to be significant effects (GLM model II, $p \leq 0.0232$; see Suppl. Table S1 for further details), and all four oils could be differentiated (Q = 2.57408, α = 0.05), as well as DI water from the Tris aqueous layer conditions (Q = 2.34774, α = 0.05). When the full data set was analysed by HCA, surfactant behaviours were clustered into 6 major groups of 2 – 7 strains which suggests that the 25 pseudomonads examined here are likely to be producing 6 or more structurally-distinct surfactants (Figure 3; see Suppl. Figure S2 for HCA constellation plots based on various combinations of assay data and Suppl. Table S4 for the mean data-set used in this analysis). However, we note that some HCA terminal (short) branches may not effectively discriminate between some surfactants, as in a preliminary pH and NaCl profiling of semi-

purified surfactants produced by DBG-1 – 4 we could only differentiate between DBG-2 and DBG-4 (GLM model III; see Suppl. Table S1 for further details; $Q = 2.6077$, $\alpha = 0.05$). Furthermore, in this HCA constellation plot, DBG-20 and DBG-24 clustered with the control strains. While DBG-20 and DBG-24 both express surfactants as assessed by surface tension measurements of culture supernatants, they clearly performed poorly in other assays used to determine surfactant activity and behaviour. We speculate that the surfactants they produce have particularly short hydrophobic tails which limit their ability to interact with hydrocarbons and result in poor oil displacement behaviours.

We have also examined pair-wise correlations between the surface tension, oil displacement, emulsion and foam stability data which supports earlier but limited comparisons of surface tension measurements and oil displacement behaviours for smaller collections of surfactant-producing strains (e.g. Yussef *et al*. 2004; Afshar *et al*. 2008). Of the 120 pair-wise correlations undertaken here, 52 were significant ($p < 0.05$) with 39 occurring within oil displacement assays and suggesting that surfactants were responding similarly to the different oils and aqueous layer conditions, and the remaining significant correlations occurring between assays which tested more diverse behaviours (see Suppl. Table S5 for pair-wise correlations). Further inspection of these correlations could be used to identify those with unexpected behaviours which might reflect significant structural variations.

Finally, a comparison of the HCA grouping of strain phenotypes and surfactant behaviours indicates that some minor clusters are conserved in both comparisons, and this suggests that closely-related strain pairs may have conserved surfactant synthesis genes and produce the same compounds (see Suppl. Table S6 for HCA grouping of strains).

3.4. Identification of putative surfactant genes in the DBG-1 draft genome

As part of a longer–term project we intend to determine the genome sequences of key pseudomonads and identify potential surfactant biosynthesis genes and predict the chemical nature of the compounds they produce. We now have a draft genome sequence for DBG-1, and our manual inspection of the CDS annotations identified seven non-ribosomal protein synthase (NRPS) genes in three clusters which may be involved in the synthesis of a cyclic lipopeptide (CLP)-like surfactant (Table 1). We were initially distracted by these annotations as they suggested that DBG-1 may produce gramicidin or tyrocidine–like antibiotics first described for the gram-negative bacterium *Bacillus brevis* (Marahier *et al*. 1993), though neither of these two antibiotics are reported to have liquid surface tension-reducing activities or otherwise considered to be surfactants.

We undertook a more sophisticated search using antiSMASH (Weber *et al.* 2015) which identified the same CDSs and predicted that the second cluster CDS might produce a CLP similar to orfamide, putisolvin, syringomycin and tolaasin, all of which are known to be produced by pseudomonads

(Raaijmakers *et al*. 2010) (Figure 4). AntiSMASH also identified the modular structure of these NRPSs which include adenylation, thiolation, and condensation domains responsible for the incorporation of each amino acid in the peptide chain (Strieker *et al*. 2010). It is noteworthy that NRPS genes contributing to the same cyclic lipopeptide are sometimes distributed in clusters across pseudomonad genomes (e.g. *P. fluorescens* SBW25 & SS101; De Bruijn *et al*. 2007 & 2008) and this may also occur in DBG-1. However, it is unclear whether DBG-1 produces a single surfactant, multiple structural analogues of one type or several different surfactant types, as metabolic analyses of surfactant–producing bacteria have revealed considerable complexity within single strains (Raaijmakers *et al*., 2010), and some NRPS are functionally active as monomers (Sieber *et al*. 2002). Further genetic analyses and biochemical characterisation will be required to properly identify the surface-active compounds produced by DBG-1.

3.5. Concluding comment

This analysis of soil-isolated *Pseudomonas* spp. strains producing high-strength surfactants has confirmed earlier predictions of the limit to the reduction of liquid surface tension that bacterial surfactants can achieve in aqueous solutions, and has shown that there is significant behavioural diversity amongst these surface-active compounds. We have begun to investigate the genetic basis of surfactant production in these strains by determining the draft genome sequence for DBG-1 and identifying potential CLP–like surfactant synthesis genes, and have recently submitted a further 10 strains for sequencing to allow further comparison within this group of pseudomonads.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

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Figure Legends

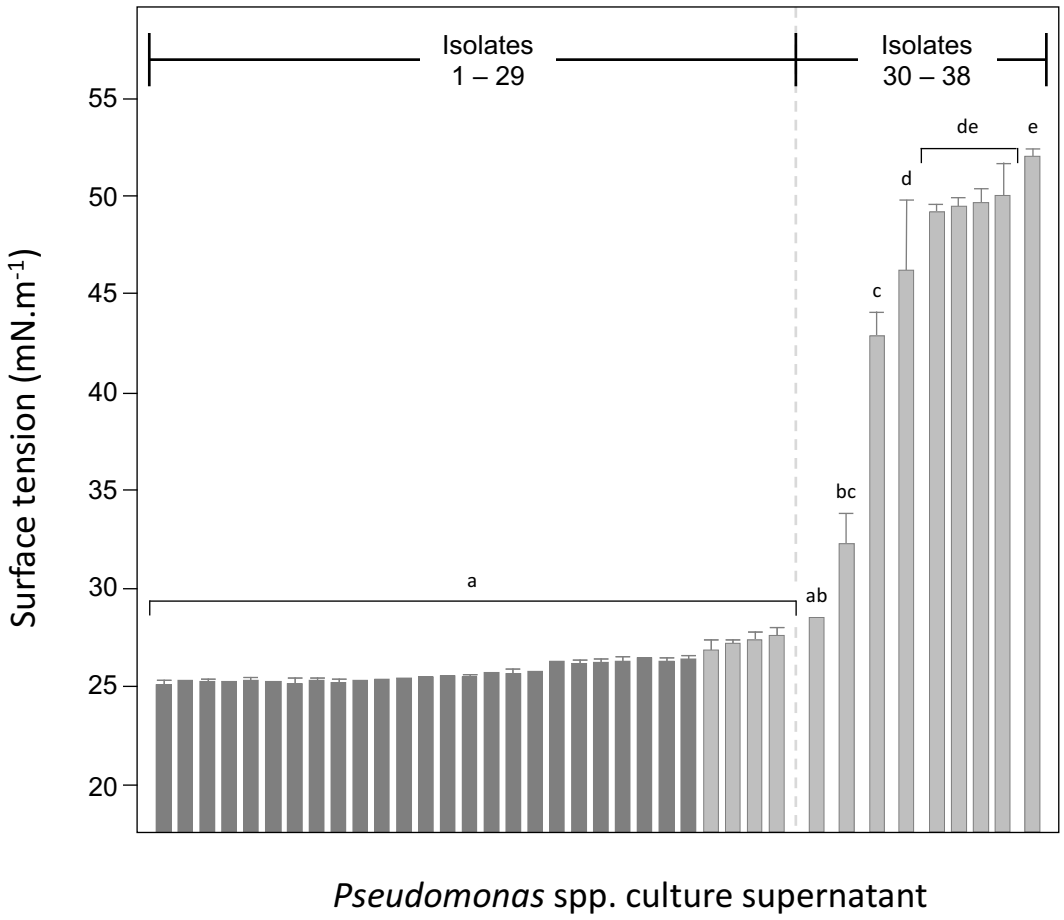
Figure 1. **Identification of a group of a homogeneous group of pseudomonads producing high-strength surfactants.** A statistical approach was taken to identify 25 Dundee Botanic Garden (DBG) strains producing high-strength surfactants. Differences between mean liquid surface tension measurements of 24 h KB* culture supernatants were determined by a Tukey-Kramer HSD test, and DBG-1 – 29 were found to form a single homogeneous group ($\alpha = 0.05$). From these, the first 25 strains (dark grey) were chosen for further analysis. Means ± SE are shown ($n = 4$), and means not linked by the same letter are significantly different ($q^* = 3.970$, $\alpha = 0.05$). The ST of sterile KB* was 52.9 ± 0.4 $mN.m^{-1}$ (not shown).

Figure 2. **The DBG pseudomonads producing high-strength surfactants are phenotypically diverse.** Hierarchical cluster analysis (HCA) was used to determine similarities between Dundee Botanic Garden (DBG) strain phenotypes using biochemical, growth, and behaviour-based assays. Shown here is a HCA constellation plot which clusters similar strains in terminal (short) branches and links strains with greater differences with longer branches. The plot is arbitrarily rooted mid-way along the longest branch (circled) and six major groups (grey arcs) determined automatically. Strains producing surfactants with the highest strength, DBG-1 – 5, are indicated by black circles, and the remainder of the surfactant–producing strains, DBG-6 – 25, are indicated by the grey circles. Non-surfactant-producing DBG-c1 – c5 control strains are indicated by white circles. Those strains that have been identified as likely *Pseudomonas* spp. are shown underlined.

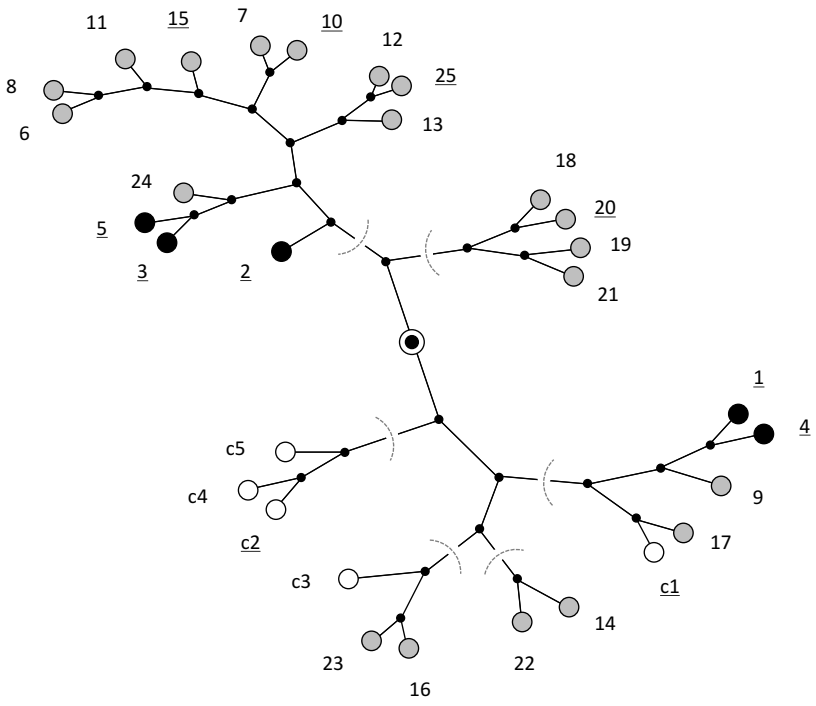
Figure 3. **Considerable behavioural diversity exists amongst the high-strength surfactants.** Hierarchical cluster analysis (HCA) was used to determine similarities between surfactant behaviours produced by Dundee Botanic Garden (DBG) strains using emulsion, foam stability, and oil displacement assays. Shown here is a HCA constellation plot which clusters surfactants with similar behaviours in terminal (short) branches and links surfactants with greater differences with longer branches. The plot is arbitrarily rooted mid-way along the longest branch (circled) and the six major groups (grey arcs) determined automatically with the limit being set by the requirement to group all of the non-surfactant-producing control strains together. Strains producing surfactants with the highest strength, DBG-1 – 5, are indicated by

black circles, and the remainder of the surfactant-producing strains, DBG-6 – 25, are indicated by the grey circles. Non-surfactant-producing DBG-c1 – c5 control strains are indicated by white circles. Those strains that have been identified as likely *Pseudomonas* spp. are shown underlined.

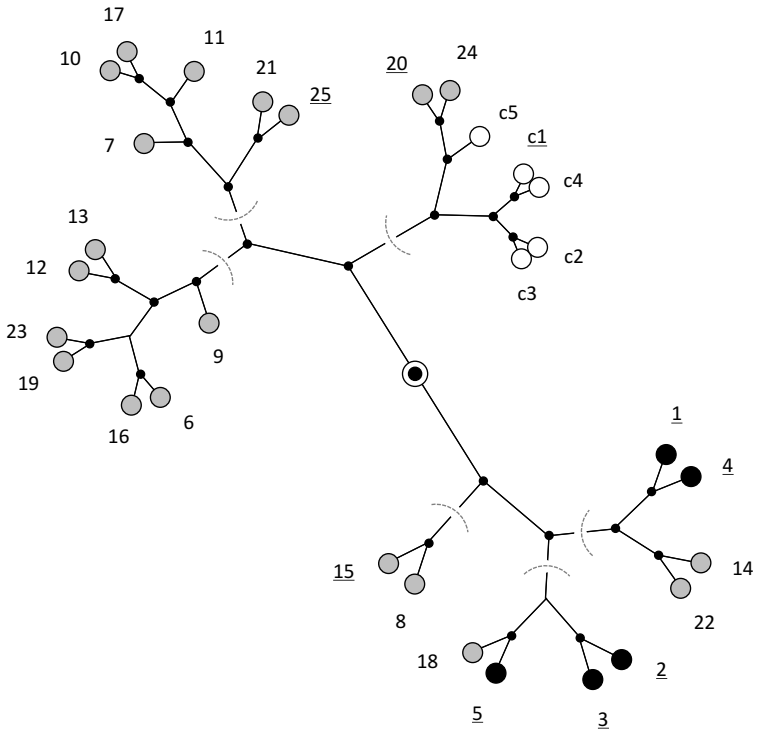
Figure 4. **Three NRPS genes in DBG-1 may be involved in surfactant production.** Inspection of the *Pseudomonas* spp. DBG-1 draft genome has identified three clusters of non-ribosomal protein synthase (NRPS) genes which may be involved in surfactant production. Shown here is Cluster II containing three NRPS genes (CDS 03215 – 03217; black) plus genes predicted to have additional biosynthetic and transport-related functions (dark grey) or roles in regulation (light grey), and unrelated genes (white) (A).Within the three NRPS genes, modules consisting of an adenylation domain (white square), thiolation domain (black oval), and condensation domain (grey circle) involved in the elongation of the peptide chain can be identified, and in CDS 03215 two terminal thioesterase domains are also present (light grey ovals) (B). Predicted gene functions and domain structures shown here are from antiSMASH analysis.



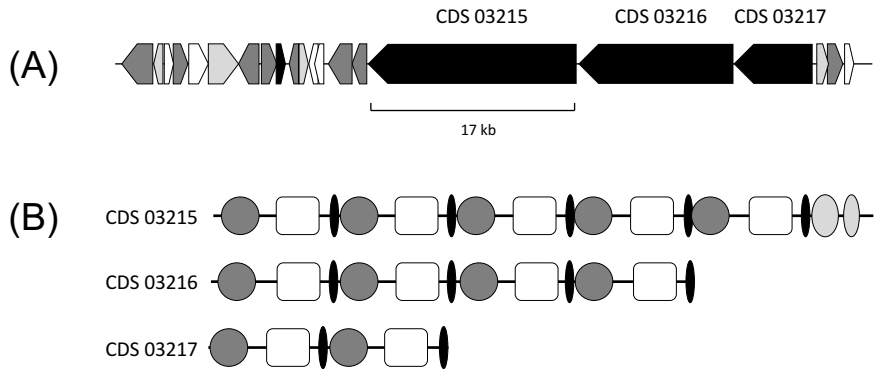
Kabir *et al*. Figure 1



Kabir *et al*. Figure 2



Kabir *et al*. Figure 3



Kabir *et al*. Figure 4

Table 1. Potential NRPS genes involved in surfactant production identified in the DBG-1 draft genome.

	CDS	Name	Size (aa)	MW	NRPS homologue	First 50 aa for identification	Similar biosynthetic gene clusters (% similar)	Predicted product
Cluster I	02131	lgrB_1	4106	456017.62	Gramicidin synthase subunit B	VQELIESVGQLSAKQRKALAVLLKQKGVNLFDIAPVFKRTAEEPLLLSYA	Pyoverdine (20%)	(thr-X-X) +
	02132	lgrB_2	2608	284684.97	Gramicidin synthase subunit B	MSGTMAERIAKRFVGLPLEQRRLFLAKLREDGKDFSLLPLPVSRHDIAPI		(asp-X) +
	02133	lgrB_3	1133	124304.305	Gramicidin synthase subunit B	MNAADAQKLARRFIELPQDKRRLFLAGMAREGIDFAQLPMTACDGIAERD		(X)
Cluster II	03215	tycC_1	5947	650,034.6	Tyrocidine synthase 3	VNVLELLATLKTKDIQLAVTDEQLRVNGNKQALSDPALLAALREHKPALI	Orfamide, putisolvin,	(d-val-gln-d-val-ile-glu) +
	03216	grsB	4338	474,976.94	Gramicidin S synthase 2	MHFSELMAAISTRAIRLQQEDEDLVILGSDDALDDALWDSLAAHKAQLLE	syringomycin, tolaasin	(thr-leu-d-val-ser) +
	03217	tycC_2	2136	235,087.06	Tyrocidine synthase 3	MQPTSANVADDVSVPVETFALTAAQRDIWLDQLSRGDSPLYNIGGYVELS	(47 – 52%)	(d-leu-asp)
Cluster III	04978	lgrB_4	4332	480,759.78	Gramicidin synthase subunit B	MTDAFELPSTLVQALQRRAALTPDRLALRFLAENEEQAVVLSYRELDERA	Pyoverdine (17%)	(X-glu-arg-ala)

CDS numbers, names, sizes and MW, and NRPS homologues are those provided in the first draft genome release, and no cluster crosses contig (node) boundaries. Non-ribosomal protein synthase (NRPS) homology confirmed by independent BLAST analyses. These clusters were also identified by antiSMASH (additional associated genes involved in biosynthesis, transport or regulation are not listed here). Low levels of similarity to known biosynthetic clusters may suggest that the DBG-1 clusters produce different compounds. antiSMASH predicted products are based on assumed NRPS colinearity without tailoring reactions taken into account (X, unidentified amino or carboxylic acid).

FEMS Microbiology Letters Special Edition on *Pseudomonas*

Uncovering behavioural diversity amongst high-strength *Pseudomonas* spp. surfactants at the limit of liquid surface tension reduction

Kamaluddeen Kabir, Yusuf Y. Deeni, Simona M. Hapca, Luke Moore & Andrew J. Spiers

Supplementary Information

S2. Supplementary Materials and Methods

S2.1. Phenotype assays

Phenotypes were determined using standard biochemical, growth, and behaviour-based assays at 20 – 22 °C (after Robertson *et al.* 2013). All assays were undertaken with replicates (n = 3 – 4) and the variation between strains allowed a ready identification between positive results (defined here) and negative results (all other outcomes). 24 h shaken KB* cultures and DI–washed cells were used to inoculate plates or cultures.

Briefly, catalase activity was assessed by mixing colony samples with hydrogen peroxide (H_2O_2) and the production of bubbles after 10 s recorded as a positive result. Oxidase activity was assessed by adding 10µl of a 1% (w/v) TMPD (N, N, N', N'-tetramethyl-p-phenylenediamine) solution to a colony and the development of a blue-purple colour within 10 s recorded as a positive result. Mucoid colonies were assessed on KB* plates after 48 h. The secretion of yellow-green fluorescent siderophore was assessed using KB* plates after 48 h. Gelatinase activity was assessed using Nutrient broth (Oxoid, UK) solidified with 120 g/L gelatine (Dr Oetker, UK) and a positive result was recorded if the inoculation site was liquidised after 48 h. Lipase activity was assessed using Tributyrin plates (Sigma-Aldrich, UK) and a positive result recorded if a clear halo was observed around the colony after 48 h. Protease (caseinase) activity was assessed using Milk agar plates (2% (w/v) dried skimmed milk powder, 0.15% (w/v) yeast extract, 1.5% (w/v) agar) and a positive result recorded if a clear halo was observed around the colony after 48 h. Sensitivity to antibiotics was tested using M13, M14 and M51 antibiotic disks (Mast Group Ltd, UK) on KB* plates, and a positive result (i.e. sensitive) recorded if no zone of inhibition was observed after 48 h. Sensitivity to mercury was assessed using KB* plates containing 10 µg/mL H_gCl_2 and colony growth assessed after 48 h. Salt tolerance was assessed using modified LB (Sambrook *et al.* 1989) plates containing 4% (w/v) NaCl and colony growth assessed after 48 h. Growth on glucose and sucrose as the sole nutrient was

assessed using DI-washed cells and minimal M9 Medium supplemented with 20 mM glucose or sucrose (Sambrook *et al.* 1989) and colony growth assessed after 48 h. KB* culture acidity was assessed by adding 5 µL of 0.1% (w/v) bromocresol green to 50 µL of overnight and a positive result recorded if the mixture remained dark. Swimming motility was assessed using KB* plates with 0.1x normal levels of nutrients and containing 0.3% (w/v) agar and a positive result recorded if an expanding ring of cells was seen around the inoculation site after 48 h. Twitching motility was assessed using KB* plates containing 1% (w/v) agar and a positive result recorded if an expanding area of growth was seen around the inoculation site between the base of the petri dish and the agar after 48 h. Swarming motility was assessed with freshly–prepared KB* plates containing 0.5% (w/v) agar and a positive result recorded if colonies developed with very irregular edges after 48 h.

S2.2. Partial 16S rDNA sequencing

In order to obtain 16S rDNA sequences, genomic DNA was isolated from over-night KB* cultures (Isolate II Genomic DNA Kit, Bioline, UK) and the 16S region amplified by PCR using as previously described (Widmer *et al.*, 1998). The resulting amplicons were cleaned and cloned into pCR2.1 (Isolate II PCR & Gel Kit, Bioline; Ligase & Topo Cloning Kit, Invitrogen, UK). Recombinant plasmid DNA was isolated (Isolate II Plasmid Mini Kit, Bioline), digested with *Eco*RI and examined by TBE agarose electrophoresis, and successful clones subjected to Sanger sequencing using SP6 and T7 primers by DNA Sequencing and Services (University of Dundee, UK). DNA trace files were examined using 4Peaks (Nucleobytes.com) and the over-lapping SP6 and T7 sequences subjected to BLASTN analysis against the 16S ribosomal RNA sequences (Bacteria & Archeae) database (ncbi.nlm.nih.gov/Blast.cgi) to identify the closest homologue. The partial 16S rDNA sequences obtained here are available on request.

S2.3. Emulsion assay

The emulsion assay was carried out at 20 – 22 °C using Diesel (Shell, UK) and replicate (n = 3) KB* cultures after Cooper & Goldenberg (1987). Vials containing 5 ml DI water and 5 ml Diesel were prepared, and 500 µl 24 h KB* culture added before mixing vigorously for 2 min. The mixtures were allowed to stand for 24 h before the height of the emulsion layer was measured (mm). The emulsion index (E_i) was calculated as the height of the emulsion / total height.

S2.4. Foam stability assay

The foam stability assay was carried out at 20 – 22 °C using replicate (n = 3) 24 h KB* cultures after Coffmann & Garcia (1977). These were vortexed vigorously for 30 s to generate foam and the initial foam height (H_i, mm) measured. The cultures were allowed to stand for 2 h before the final foam height (H_f) was measured. The percentage foam reduction was determined as 100 x (H_i – H_f) / H_i.

S2.5. Oil displacement assays

The oil displacement assay was carried out at 20 – 22 °C using Mineral oil (Sigma-Aldrich, UK), Vegetable oil (Tesco, UK), Diesel (Shell, UK) and Used lubricating oil (ULO, obtained from a local garage) and replicate (n = 3) 24 h KB* cultures after Morikawa *et al.* (1993). Petri dishes containing 40 ml DI water (pH 6), 200 mM NaCl (pH 6) or 50 mM Tris (pH 8) were prepared and 10 µl of Mineral oil, ULO or Diesel (or 100 µl Vegetable oil) added to form a thin layer at the surface. 10 µl of culture was then added and the diameter of the oil-free zone was measured (mm) after 5 s.

S2.6. Critical micelle concentrations (CMC) and pH and NaCl surface tension profiles

In order to confirm that semi-purified surfactants re-suspended in DI water were at a concentration above the critical micelle concentration (CMC), replicate samples (n = 3) were sequentially diluted and surface tension measurements made until a significant increase in surface tension was observed (i.e. the surfactants had been diluted below the CMC). pH and NaCl surface tension profiles of semi-purified surfactants re-suspended in DI water were determined from replicate samples (n = 3) to which citrate, glycine, and phosphate buffers had been added to alter pH to pH 4, 6, 8, 10 and 12, and NaCl added to 200, 400, 600, 800 and 1000 mM before surface tension measurement.

Supplementary Information References

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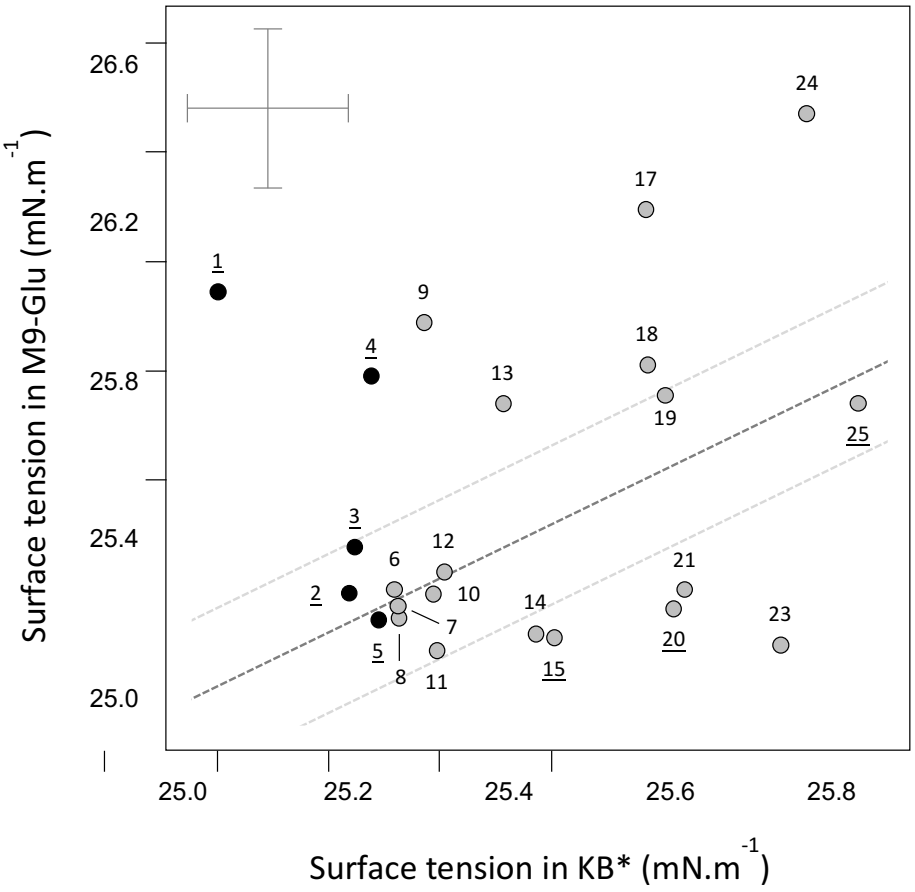
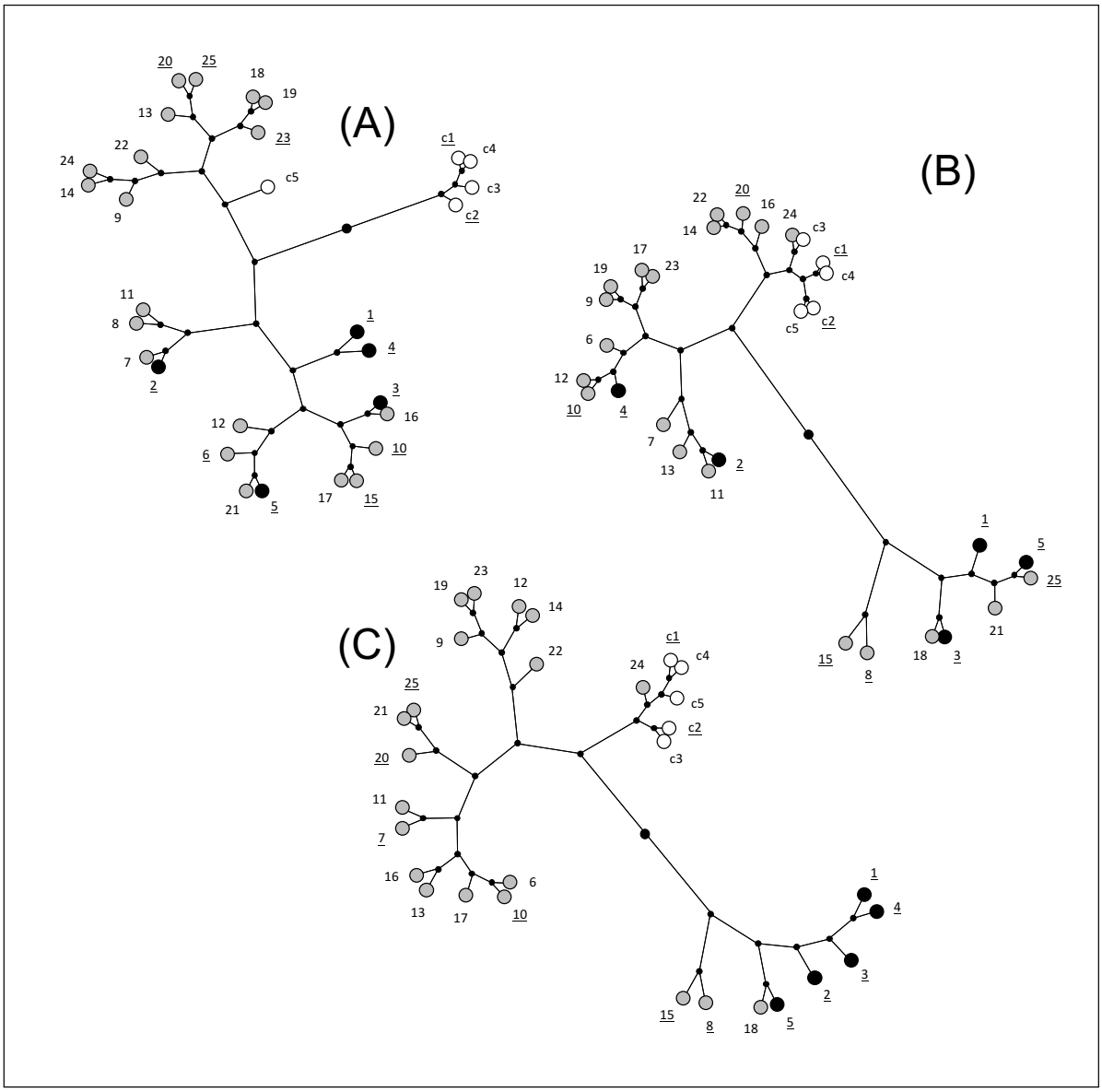
Supplementary Figures (Fig. S1 – S2)



Suppl. Figure S1. Comparison between surface tension measurements made from different culture supernatants suggest the surfactant activities are influenced by buffer compositions.

Surfactant activities for the 25 Dundee Botanic Garden (DBG) strains producing surfactants were determined by liquid surface tension measurements of KB* and M9-Glu culture supernatants. Shown here are the means (circles) (n = 4; SE are omitted for clarity but the pooled SE for all measurements of 0.36 mN.m^{-1} is shown by the cross in the top left of the figure), with strains positioned above the dashed line showing higher than expected surface tension activity in M9-Glu, and those under the line showing lower than expected activity in M9-Glu, compared to KB*. Note however that no significant correlation was seen between KB* and M9-Glu surface tension measurements (r = 0.24; p = 0.27). The surface tension of sterile KB* and M9Glu culture media was 52.9 ± 0.4 and 70.7 ± 0.7 mN.m^{-1}, respectively. Surfactant-producing DBG-1 – 5 and 6 – 25 strains are indicated by black and grey circles, respectively, and those identified as likely *Pseudomonas* spp. are underlined.



Suppl. Figure S2. Similarities in surfactant behaviour determined by different assays.

Hierarchical cluster analysis (HCA) was used to determine similarities between surfactant behaviours produced by Dundee Botanic Garden (DBG) strains using different combinations of emulsion, foam stability, and oil-displacement assays. Shown here are the HCA constellation plots produced using the foam stability assay, Diesel emulsion, and Diesel displacement assays overlaying deionised (DI) water (pH 6) (A); Mineral oil displacement assays overlaying DI water (pH 6), 200 mM NaCl (pH 6) and 50 mM Tris (pH 8) solutions (B); and Mineral oil, Vegetable oil, ULO, and Diesel displacement assays overlaying DI water (pH 6) (C). Shown here are HCA constellation plots drawn to the same scale which cluster surfactants with similar behaviours in terminal (short) branches and link surfactants with greater differences with longer branches. The plots are arbitrarily rooted mid-way along the longest branch (circled). Strains producing surfactants with the highest strength, DBG-1 – 5, are indicated by black circles, and the remainder of the surfactant-producing strains, DBG-6 – 25, are indicated by the grey circles. Non-surfactant-producing DBG-c1 – c5 control strains are indicated by white circles. Those strains that have been identified as likely *Pseudomonas* spp. are shown underlined.

Supplementary Tables (Table S1 – S6)

Table S1. Statistical Models and Effects Tests.

I Surface tension (ST) as variable with Strain [a], Assay environment and Replicate as co-variates ($F_{16, 160}$ = 31.1127, p < 0.0001).

Effects tests :	Source	Nparm	DF	F Ratio	Prob > F
	Strain	13	13	4.2857	< 0.0001
	Assay environment	2	2	206.2452	< 0.0001
	Replicate	1	1	0.5810	0.4470

II Oil–displacement as variable with Strain [b], Oil type, Aqueous layer conditions, and Replicate as co-variates ($F_{30, 867}$ = 25.5307, p < 0.0001).

Effects tests :	Source	Nparm	DF	F Ratio	Prob > F
	Strain	24	24	26.9426	< 0.0001
	Oil type	3	3	36.6524	< 0.0001
	Aqueous layer conditions	2	2	3.7813	0.0232
	Replicate	1	1	1.4261	0.2327

III Surface tension (ST) as variable with Strain [c], NaCl concentration, pH, and Replicate as co-variates ($F_{6, 8113}$ = 9.326, p < 0.0001).

Effects tests :	Source	Nparm	DF	F Ratio	Prob > F
	Strain	3	3	3.1426	0.0281
	NaCl concentration	1	1	26.6767	< 0.0001
	pH	1	1	4.8779	0.0292
	Replicate	1	1	0.0486	0.8260

[a] DBG-1, 2, 3, 4, 5, 7, 10, 11, 14, 15, 16, 20, 21 and 25; [b] DBG-1 –25 (DBG-c1 – c5 excluded from this analyses as the control results were always substantially different from the surfactant-producing strains, data not shown); [c] DBG-1 – 4.

Table S2. Dundee Botanic Garden (DBG) strain phenotypes.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
DBG-1	p	n	p	p	p	p	p	p	p	p	p	n	p	p	p	n	n	p	p	n	n	p	n	n
DBG-2	n	p	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	n	p	n	n	p	n	n
DBG-3	n	p	p	p	p	p	p	p	p	p	p	n	p	n	p	n	n	p	p	n	p	n	n	n
DBG-4	p	n	p	p	p	p	p	p	p	p	p	n	p	p	p	n	n	p	n	n	n	p	n	p
DBG-5	n	p	p	p	p	p	p	p	p	p	p	n	p	n	p	n	n	p	p	n	p	n	n	p
DBG-6	n	p	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	p	p	n	p	n	n	p
DBG-7	n	p	p	p	p	p	p	n	n	p	p	n	p	n	p	n	n	p	p	n	n	n	n	p
DBG-8	n	p	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	p	p	n	p	n	n	n
DBG-9	n	n	p	p	p	p	p	n	p	p	p	p	p	p	p	n	n	p	p	n	p	p	n	n
DBG-10	n	p	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	p	p	n	n	n	n	p
DBG-11	n	p	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	p	n	n	p	n	n	p
DBG-12	n	n	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	p	p	n	p	n	n	p
DBG-13	n	n	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	p	p	n	p	n	n	n
DBG-14	n	p	p	n	p	p	p	n	p	n	p	p	n	n	p	n	p	p	p	p	p	p	n	n
DBG-15	n	p	p	p	p	p	p	p	n	p	p	p	p	n	p	n	n	p	p	n	p	n	n	p
DBG-16	p	p	p	p	p	p	n	p	p	p	p	p	p	n	p	n	n	p	p	n	n	p	n	p
DBG-17	p	n	p	p	p	p	p	p	p	n	p	p	p	p	p	n	n	n	p	p	n	p	n	n
DBG-18	p	p	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	p	p	n	p	n	n	n
DBG-19	p	p	p	p	p	p	p	p	p	n	p	n	p	p	p	n	n	p	p	n	p	n	n	n
DBG-20	p	p	p	p	p	p	p	p	n	p	p	n	p	p	p	n	n	p	p	n	p	p	n	n
DBG-21	p	p	p	p	p	p	p	p	p	p	p	p	p	n	p	n	n	p	p	n	p	n	n	n
DBG-22	p	p	p	n	p	p	n	p	p	p	p	p	n	p	p	n	p	p	p	p	n	p	n	n
DBG-23	p	p	p	p	p	p	n	p	p	p	p	p	p	n	p	n	n	p	p	n	p	p	n	p
DBG-24	n	p	p	p	p	p	p	p	p	n	p	n	p	n	p	n	n	p	p	n	p	n	n	p
DBG-25	n	n	p	p	p	p	p	p	n	p	p	n	p	n	p	n	n	p	p	n	p	n	n	p
DBG-c1	p	n	p	p	p	p	n	p	p	n	p	p	p	p	p	n	n	n	p	n	n	p	n	n
DBG-c2	n	n	p	p	n	n	n	p	p	n	n	p	p	n	p	n	n	p	p	p	p	n	n	p
DBG-c3	n	p	p	p	p	n	n	p	p	p	p	p	p	p	p	n	n	n	p	p	p	n	p	p
DBG-c4	n	n	p	p	n	n	n	p	p	n	n	p	p	n	p	n	n	p	p	n	p	p	n	n
DBG-c5	n	n	p	p	n	n	n	n	p	n	n	n	p	n	p	n	n	p	p	n	p	p	n	p

Assays (p, positive; n, negative) : 1, Mucoidal colony; 2, Fluorescent siderophore; 3, Catalase; 4, Oxidase, 5, Gelatinase; 6, Protease; 7, Lipase; 8, Swimming; 9, Twitching; 10, Swarming; 11, NaCl tolerance; 12, Hg sensitivity; 13, KB* culture acidity; 14, Sucrose; 15, Glucose; 16, Tetracycline resistance (plate); 17, Kanamycin resistance (plate); 18, Streptomycin (disk); 19, Tetracycline (disk); 20, Chloramphenicol (disk); 21; Naladixic acid (disk), 22, Coliston sulphate (disk); 23, Cotrimoxazole (disk); and 24, Streptomycin (disk).

Table S3. Identification of select Dundee Botanic Garden (DBG) strains.

Strain	Metabolic profile identity	16S rDNA sequence homologue
DBG-1	*Pseudomonas fluorescens* / *putida*	*Pseudomonas helmanticensis*
DBG-2	*Pseudomonas fluorescens* / *putida*	*Pseudomonas trivialis*
DBG-3	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*
DBG-4	*Pseudomonas fluorescens* / *putida*	*Pseudomonas helmanticensis*
DBG-5	*Pseudomonas aeruginosa*	*Pseudomonas salomonii*
DBG-10	*Pseudomonas luteola*	ND
DBG-15	Unidentified	*Pseudomonas fluorescens*
DBG-20	*Pseudomonas fluorescens* / *putida*	*Pseudomonas marginalis*
DBG-25	*Pseudomonas fluorescens* / *putida*	*Pseudomonas fluorescens*
DBG-c1	Unidentified	*Pseudomonas kilonesis*
DBG-c2	*Pseudomonas fluorescens* / *putida*	ND

Identification of select strains was by metabolic profiling using API 20e plates and by partial 16S rDNA sequence analysis. For the latter, the ID match for all sequences was 99% and top species listed in the BLAST reports are provided. ND, Sequence not determined.

Table S4. Dundee Botanic Garden (DBG) strain surfactant behaviours.

Strain	Emulsion (E_i)	Foam stability (% Reduction)	Oil Displacement (mm)											
			Mineral Oil			Vegetable Oil			ULO			Diesel		
			DI Water	NaCl	Tris	DI Water	NaCl	Tris	DI Water	NaCl	Tris	DI Water	NaCl	Tris
DBG-1	40.1	32.0	13.7	25.3	13.0	43.7	34.3	33.3	35.0	27.7	26.3	19.7	20.7	10.0
DBG-2	10.6	56.5	11.3	13.3	30.3	36.3	45.0	33.0	42.3	24.0	17.7	52.0	31.0	23.7
DBG-3	21.1	41.9	24.0	31.7	36.3	40.7	40.3	36.7	43.3	39.0	28.3	28.0	29.7	30.3
DBG-4	23.3	14.7	9.3	6.7	13.3	29.7	39.7	30.3	34.7	36.3	27.3	16.0	12.3	10.3
DBG-5	32.7	50.0	23.0	20.0	25.7	26.3	31.0	28.3	29.0	20.7	12.3	24.3	35.0	17.3
DBG-6	39.1	68.7	7.0	4.3	20.3	24.0	26.0	16.0	12.7	9.7	14.3	30.3	13.0	25.0
DBG-7	5.8	62.9	5.3	4.3	40.0	21.3	13.3	13.3	13.3	15.3	20.3	51.7	43.0	40.7
DBG-8	14.5	46.4	43.3	43.0	65.3	33.3	24.0	26.3	28.3	33.0	36.0	62.0	63.3	41.3
DBG-9	15.7	56.0	7.3	8.3	8.3	21.0	11.0	9.3	9.0	14.3	8.7	16.0	14.7	15.0
DBG-10	21.6	62.1	7.7	7.7	16.3	20.7	14.7	12.7	12.7	14.7	15.0	35.7	42.3	32.7
DBG-11	19.0	55.2	10.7	10.0	22.3	11.3	8.0	6.7	7.3	8.7	4.7	52.7	53.0	36.3
DBG-12	27.2	70.8	8.0	10.0	17.7	20.0	16.0	12.7	22.0	23.0	17.3	16.3	26.7	17.7
DBG-13	12.2	68.1	9.0	17.0	20.3	13.3	12.0	13.7	18.0	17.3	15.7	30.7	33.0	24.0
DBG-14	15.1	45.2	4.3	3.0	3.3	17.7	24.0	23.7	24.7	29.0	29.3	7.3	7.7	4.7
DBG-15	22.9	54.1	42.3	34.7	39.7	20.7	14.0	15.0	31.3	31.7	31.0	38.3	41.7	38.3
DBG-16	21.5	47.6	3.3	2.3	11.7	19.7	17.3	12.7	14.3	10.3	10.0	24.3	23.0	12.0
DBG-17	25.3	48.2	9.3	10.0	9.3	18.3	19.3	13.7	15.7	11.0	7.3	40.7	40.0	28.7
DBG-18	13.5	68.3	21.0	27.7	34.3	26.7	23.3	21.3	22.3	15.3	14.3	13.0	23.3	21.0
DBG-19	11.7	71.4	7.3	7.3	5.3	20.7	19.0	17.7	15.3	14.3	8.7	12.7	15.0	19.7
DBG-20	6.5	56.7	5.3	4.3	3.7	5.3	3.7	1.0	5.3	5.0	0.0	24.7	15.0	14.0
DBG-21	35.1	51.3	22.3	25.0	15.3	4.3	2.3	0.0	4.7	4.7	0.0	30.3	30.0	21.7
DBG-22	13.9	31.7	3.0	2.3	2.7	38.0	35.7	31.0	17.0	20.3	19.3	6.3	6.7	7.3
DBG-23	12.3	63.1	9.7	9.0	5.3	17.7	27.0	22.3	16.0	17.0	13.3	12.3	12.3	10.0
DBG-24	13.2	57.3	0.3	1.0	2.3	5.3	5.7	2.7	5.7	6.3	0.0	3.3	4.3	4.7
DBG-25	12.8	52.3	21.0	24.3	22.7	4.7	4.7	1.3	4.3	6.7	0.0	27.3	24.0	23.0
DBG-c1	0.0	100.0	0.0	0.0	0.3	6.7	4.3	1.7	5.3	6.7	0.0	0.0	0.0	0.0
DBG-c2	1.1	100.0	0.0	0.0	0.0	7.0	6.7	2.3	8.3	5.7	0.0	0.0	0.0	0.0
DBG-c3	0.0	100.0	1.0	2.3	0.3	6.7	7.7	3.7	8.0	4.7	0.0	1.7	1.0	0.7
DBG-c4	0.0	100.0	0.0	0.0	0.3	5.0	4.3	0.0	6.0	5.0	0.0	0.0	0.0	0.0
DBG-c5	0.0	20.8	0.0	0.0	0.0	5.7	6.7	6.0	3.7	4.0	0.0	0.0	0.0	0.0

Only means are shown. ULO, Used Lubricating Oil; NaCl, Tris and DI water refer to the different aqueous layer conditions in the oil displacement assays.

Table S5. Correlations between Surface Tension, Emulsion, Foam Stability and Oil Displacement Assays.

First Variable (Assay)	Second Variable (Assay)	Correlation	Sig. Prob.
Surface tension of M9-Glu culture supernatant	Surface tension of KB culture supernatant	0.3105	0.1308
Mineral oil displacement on a NaCl solution	Surface tension of KB culture supernatant	-0.1999	0.3382
	Surface tension of M9-Glu culture supernatant	-0.3298	0.1074
Mineral oil displacement on a Tris solution	Surface tension of KB culture supernatant	-0.4172	0.0380*
	Surface tension of M9-Glu culture supernatant	-0.3509	0.0855
	Mineral oil displacement on a NaCl solution	0.7466	<0.0001*
Mineral oil displacement on DI water	Surface tension of KB culture supernatant	-0.1522	0.4678
	Surface tension of M9-Glu culture supernatant	-0.3550	0.0816
	Mineral oil displacement on a NaCl solution	0.9372	<0.0001*
	Mineral oil displacement on a Tris solution	0.7758	<0.0001*
Vegetable oil displacement on a NaCl solution	Surface tension of KB culture supernatant	-0.4745	0.0165*
	Surface tension of M9-Glu culture supernatant	0.0907	0.6665
	Mineral oil displacement on a NaCl solution	0.1070	0.6105
	Mineral oil displacement on a Tris solution	0.1527	0.4661
	Mineral oil displacement on DI water	0.0653	0.7566
Vegetable oil displacement on a Tris solution	Surface tension of KB culture supernatant	-0.5144	0.0085*
	Surface tension of M9-Glu culture supernatant	0.0534	0.7997
	Mineral oil displacement on a NaCl solution	0.2407	0.2464
	Mineral oil displacement on a Tris solution	0.2523	0.2238
	Mineral oil displacement on DI water	0.1846	0.3772
	Vegetable oil displacement on a NaCl solution	0.9619	<0.0001*
Vegetable oil displacement on DI water	Surface tension of KB culture supernatant	-0.6139	0.0011*
	Surface tension of M9-Glu culture supernatant	0.1463	0.4853
	Mineral oil displacement on a NaCl solution	0.2801	0.1751
	Mineral oil displacement on a Tris solution	0.3347	0.1019
	Mineral oil displacement on DI water	0.2033	0.3296
	Vegetable oil displacement on a NaCl solution	0.8850	<0.0001*
	Vegetable oil displacement on a Tris solution	0.9232	<0.0001*
ULO displacement on a NaCl solution	Surface tension of KB culture supernatant	-0.5569	0.0038*
	Surface tension of M9-Glu culture supernatant	-0.1610	0.4419
	Mineral oil displacement on a NaCl solution	0.4340	0.0302*
	Mineral oil displacement on a Tris solution	0.4243	0.0345*
	Mineral oil displacement on DI water	0.4358	0.0294*
	Vegetable oil displacement on a NaCl solution	0.7104	<0.0001*
	Vegetable oil displacement on a Tris solution	0.8075	<0.0001*
	Vegetable oil displacement on DI water	0.7362	<0.0001*
ULO displacement on a Tris solution	Surface tension of KB culture supernatant	-0.5772	0.0025*
	Surface tension of M9-Glu culture supernatant	-0.1259	0.5488
	Mineral oil displacement on a NaCl solution	0.3994	0.0480*
	Mineral oil displacement on a Tris solution	0.5142	0.0085*
	Mineral oil displacement on DI water	0.4248	0.0343*
	Vegetable oil displacement on a NaCl solution	0.6301	0.0007*
	Vegetable oil displacement on a Tris solution	0.7511	<0.0001*
	Vegetable oil displacement on DI water	0.7309	<0.0001*
	ULO displacement on a NaCl solution	0.9219	<0.0001*
ULO displacement on DI water	Surface tension of KB culture supernatant	-0.6122	0.0011*
	Surface tension of M9-Glu culture supernatant	-0.1426	0.4965
	Mineral oil displacement on a NaCl solution	0.4386	0.0283*
	Mineral oil displacement on a Tris solution	0.4247	0.0343*
	Mineral oil displacement on DI water	0.3945	0.0510
	Vegetable oil displacement on a NaCl solution	0.8375	<0.0001*

	Vegetable oil displacement on a Tris solution	0.8777	<0.0001*
	Vegetable oil displacement on DI water	0.8090	<0.0001*
	ULO displacement on a NaCl solution	0.8952	<0.0001*
	ULO displacement on a Tris solution	0.7872	<0.0001*
Diesel displacement on a NaCl solution	Surface tension of KB culture supernatant	-0.3767	0.0634
	Surface tension of M9-Glu culture supernatant	-0.3355	0.1011
	Mineral oil displacement on a NaCl solution	0.5503	0.0044*
	Mineral oil displacement on a Tris solution	0.7584	<0.0001*
	Mineral oil displacement on DI water	0.5926	0.0018*
	Vegetable oil displacement on a NaCl solution	-0.1309	0.5329
	Vegetable oil displacement on a Tris solution	-0.0277	0.8953
	Vegetable oil displacement on DI water	0.0658	0.7546
	ULO displacement on a NaCl solution	0.1388	0.5083
	ULO displacement on a Tris solution	0.2276	0.2738
	ULO displacement on DI water	0.1423	0.4973
Diesel displacement on a Tris solution	Surface tension of KB culture supernatant	-0.3144	0.1259
	Surface tension of M9-Glu culture supernatant	-0.4228	0.0352*
	Mineral oil displacement on a NaCl solution	0.4928	0.0123*
	Mineral oil displacement on a Tris solution	0.7803	<0.0001*
	Mineral oil displacement on DI water	0.5585	0.0037*
	Vegetable oil displacement on a NaCl solution	-0.1600	0.4449
	Vegetable oil displacement on a Tris solution	-0.0906	0.6666
	Vegetable oil displacement on DI water	0.0426	0.8397
	ULO displacement on a NaCl solution	0.0930	0.6584
	ULO displacement on a Tris solution	0.2157	0.3005
	ULO displacement on DI water	0.0758	0.7186
	Diesel displacement on a NaCl solution	0.8813	<0.0001*
Diesel displacement on DI water	Surface tension of KB culture supernatant	-0.4115	0.0410*
	Surface tension of M9-Glu culture supernatant	-0.3438	0.0925
	Mineral oil displacement on a NaCl solution	0.3862	0.0566
	Mineral oil displacement on a Tris solution	0.7160	<0.0001*
	Mineral oil displacement on DI water	0.4461	0.0254*
	Vegetable oil displacement on a NaCl solution	-0.0534	0.7997
	Vegetable oil displacement on a Tris solution	-0.0339	0.8720
	Vegetable oil displacement on DI water	0.0732	0.7279
	ULO displacement on a NaCl solution	0.0572	0.7860
	ULO displacement on a Tris solution	0.1758	0.4006
	ULO displacement on DI water	0.1273	0.5442
	Diesel displacement on a NaCl solution	0.8843	<0.0001*
	Diesel displacement on a Tris solution	0.8662	<0.0001*
Diesel emulsion	Surface tension of KB culture supernatant	-0.3991	0.0481*
	Surface tension of M9-Glu culture supernatant	-0.0385	0.8550
	Mineral oil displacement on a NaCl solution	0.1995	0.3391
	Mineral oil displacement on a Tris solution	-0.0326	0.8770
	Mineral oil displacement on DI water	0.1767	0.3981
	Vegetable oil displacement on a NaCl solution	0.1811	0.3863
	Vegetable oil displacement on a Tris solution	0.1419	0.4987
	Vegetable oil displacement on DI water	0.2204	0.2899
	ULO displacement on a NaCl solution	0.1003	0.6335
	ULO displacement on a Tris solution	0.0962	0.6475
	ULO displacement on DI water	0.1844	0.3777
	Diesel displacement on a NaCl solution	0.0396	0.8509
	Diesel displacement on a Tris solution	-0.0436	0.8360
	Diesel displacement on DI water	-0.0314	0.8817
Foam stability	Surface tension of KB culture supernatant	0.1959	0.3479
	Surface tension of M9-Glu culture supernatant	-0.3254	0.1125
	Mineral oil displacement on a NaCl solution	-0.0971	0.6442
	Mineral oil displacement on a Tris solution	0.0689	0.7434
	Mineral oil displacement on DI water	-0.1002	0.6338
	Vegetable oil displacement on a NaCl solution	-0.4703	0.0177*
	Vegetable oil displacement on a Tris solution	-0.4660	0.0189*
	Vegetable oil displacement on DI water	-0.4298	0.0320*

ULO displacement on a NaCl solution	-0.4935	0.0122*
ULO displacement on a Tris solution	-0.3798	0.0611
ULO displacement on DI water	-0.4047	0.0448*
Diesel displacement on a NaCl solution	0.1217	0.5622
Diesel displacement on a Tris solution	0.2804	0.1745
Diesel displacement on DI water	0.0994	0.6365
Diesel emulsion	-0.2310	0.2665

* Indicates significant correlations between means for DBG-1 – 25 ($p < 0.05$) (DBG-c1 – c5 excluded from this analyses as the control results were always substantially different from the surfactant-producing strains, data not shown); ULO, Used Lubricating Oil; NaCl, Tris and DI water refer to the different aqueous layer conditions in the oil displacement assays.

Table S6. Strain Phenotype and Surfactant Behaviour HCA Groups.

Strain	P	B	Strain	P	B	Strain	P	B
DBG-1	3	2	DBG-11	1	6	DBG-21	2	6
DBG-2	1	3	DBG-12	1	5	DBG-22	4	2
DBG-3	1	3	DBG-13	1	5	DBG-23	5	5
DBG-4	3	2	DBG-14	4	2	DBG-24	1	1
DBG-5	1	3	DBG-15	1	4	DBG-25	1	6
DBG-6	1	5	DBG-16	5	5	DBG-c1	3	1
DBG-7	1	6	DBG-17	3	6	DBG-c2	6	1
DBG-8	1	4	DBG-18	2	3	DBG-c3	5	1
DBG-9	3	5	DBG-19	2	5	DBG-c4	6	1
DBG-10	1	6	DBG-20	2	1	DBG-c5	6	1

Independent HCA was used to cluster Dundee Botanic Garden (DBG) strains into 6 groups using the strain phenotype (P) and surfactant behaviour (B) data (the later restricted by the requirement to cluster all of the control strains into the same group). Strains within the same group in both analyses are likely to be phylogenetically-related strains carrying similar surfactant synthesis genes and producing structurally-related surfactants (e.g. DBG-2, 3 & 5; DBG-6, 12 & 13; etc.).